Anal Bioanal Chem (2008) 390:2123–2132
DOI 10.1007/s00216-008-1966-3

ORIGINAL PAPER

Comparison of methods with respect to efficiencies, recoveries, and quantitation of mercury species interconversions in food demonstrated using tuna fish

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Received: 8 December 2007 / Revised: 6 February 2008 / Accepted: 9 February 2008 / Published online: 4 March 2008

Abstract Eight different analytical extraction procedures commonly used to extract mercury species from biological samples were evaluated by analyzing Tuna Fish Tissue Certified Reference Material (ERM-CE464) certified for the content of total mercury and methylmercury. Speciated isotope dilution mass spectrometry (SIDMS; US Environmental Protection Agency's method 6800) was utilized to evaluate and effectively compensate for potential errors during measurement and accurately quantify mercury species using all the extraction methods. SIDMS was used to accurately evaluate species transformations during sample pretreatment, preparation and analysis protocols. The extraction methods tested in this paper were based on alkaline extraction with KOH or tetramethylammonium hydroxide; acid leaching with HCl, HNO_3 or CH_3COOH; extraction with L-cysteine hydrochloride; and enzymatic digestion with protease XIV. Detection of total mercury and mercury species from all extraction methods was carried out by inductively coupled plasma mass spectrometry (ICP-MS) and high-performance liquid chromatography–ICP-MS, respectively. Microwave-assisted extraction and ultrasound-assisted extraction were found to be the most efficient alkaline digestion protocols that caused the lowest levels of transformation of mercury species (6% or less). Extraction with 5 M HCl or enzymatic digestion with protease resulted in the second-highest extraction efficiency, with relatively lower transformation of methylmercury to inorganic mercury (3 and 1.4%, respectively). Despite frequent use of acid leaching for the extraction of mercury species from tuna fish samples, the lowest extraction efficiencies and the highest mercury species transformation were obtained when microwave-assisted extraction with 4 M HNO_3 or CH_3COOH was used. Transformations as high as 30% were found using some literature protocols; however, all the extractions tested produced accurate quantitation when corrected in accordance with the SIDMS method standardized in the US Environmental Protection Agency's method 6800.

Keywords Mercury speciation · Speciated isotope dilution mass spectrometry · EPA method 6800 · Extraction · High-performance liquid chromatography–inductively coupled plasma mass spectrometry · Tuna fish

Introduction

Mercury (Hg) is one of the most hazardous pollutants in the environment. It is introduced into the environment in several major forms: elemental mercury (Hg^0), inorganic mercury (Hg^{2+}) and organic mercury species [1]. Hg^{2+} and methylmercury (CH_3Hg^+) are the two major mercury species found in environmental and biological samples. CH_3Hg^+ is one of the most toxic mercury species found in the environment. CH_3Hg^+ can be formed naturally by biomethylation of Hg^{2+} in the aquatic environment and is bioaccumulated through aquatic food chains. Total mercury concentrations can reach up to 4,000 μg/kg consisting up to 95% CH_3Hg^+ in large predatory fish, such as shark, king mackerel, swordfish and some mature tuna species [2, 3].

CH_3Hg^+ is efficiently adsorbed from the gastrointestinal tract. It passes the blood–brain and placenta barriers, and is neurotoxic as well as teratogenic. For humans and wildlife, fish consumption is the major contributor to mercury risk

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[4]. The devastating effects of CH_3Hg^+ were recognized in the 1960s when consumption of contaminated fish in Minamata, Japan, resulted in neurological damage, including cerebral palsy and death [1, 4]. Dietary popularity around the world and increasingly high levels of CH_3Hg^+ in certain types of fish have led regulatory agencies to focus on fish as the target organism to protect the health of humans and other sensitive organisms [4]. Subsequently, the Environmental Protection Agency (EPA) has established a reference dose at 0.1 μg/kg body weight/day, and the World Health Organization has set the tolerable weekly dose at 1.6 μg/kg body weight/week (0.23 μg/kg body weight/day) for CH_3Hg^+ [5]. For these reasons, it is now essential to routinely monitor and evaluate the chemical risks derived from fish consumption. Speciation analysis of mercury is, therefore, mandatory in environmental studies, not only for the understanding of the mercury biogeochemical cycle, but also for the accurate determination of toxicity levels.

Numerous analytical methods have been described in the literature for the measurement of mercury species in biological samples. Analytical determinations of mercury species are usually carried out by hyphenated techniques, including gas chromatography (GC) or high-performance liquid chromatography (HPLC) coupled with a mercury-selective detection technique [6–15] (G.M.M. Rahman, T. Fahrenholz and H.M. Kingston, unpublished results). In comparison with GC, HPLC has the advantage of simplified sample preparation [16]. The detection methods coupled with HPLC for mercury speciation include atomic fluorescence spectrometry [6–8], atomic emission spectrometry [10], atomic absorption spectrometry [9] and inductively coupled plasma mass spectrometry (ICP-MS) [14, 15]. Among the methods mentioned above, the coupling of HPLC with ICP-MS appears to be one of the most common methods for mercury speciation analysis because of the simplicity of the interface. In addition to high sensitivity and selectivity, ICP-MS offers the opportunity to perform speciated isotope dilution mass spectrometry (SIDMS); this has the highest number of literature references over the past several decades. Not only is this technique highly accurate and precise, but it can also permit correction for species transformations and extraction recoveries by using isotopically enriched species analogues as tracers [16, 17]. Use of SIDMS has been reported in publications for mercury speciation for biological samples [18–22] (G.M.M. Rahman, T. Fahrenholz and H.M. Kingston unpublished results). Most of these studies were essentially focused on the analyses of tuna fish samples.

Despite the significant progress in the quality of mercury analysis instruments, reliable results are still mainly dependent on sample preparation. There are three approaches to quantitative speciated measurements of reactive species. One desirable method is an extraction procedure for speciation analysis with high extraction efficiency and, more importantly, species stability [16]. Alternatively the correction of species transformation is necessary as described in EPA method 6800 [17]. Usually, alkaline leaching with either strong or weak bases, e.g., methanolic potassium hydroxide or tetramethylammonium hydroxide (TMAH) [11, 12], or acid leaching [11, 13, 19] (G.M.M. Rahman, T. Fahrenholz and H.M. Kingston, unpublished results) is used to separate mercury species from biological tissues. Additionally, some leaching solutions such as 0.1% (w/v) L-cysteine [14], 0.1% (v/v) HCl/0.1% (v/v) 2-mercapthoethanol/0.15% (w/v) KCl [23] or diluted 5–20% (v/v) HNO_3/0.02–0.2% (v/v) thiourea [24] have also been reported for the extraction of mercury species. Furthermore, several enzymatic digestion protocols involving trypsin [25], protease XIV [15, 26] and lipase [26] have been used in mercury speciation studies. Room-temperature or elevated-temperature procedures using conventional heating [14, 15, 26], microwave-assisted extraction [12, 13] (G.M.M. Rahman, T. Fahrenholz and H.M. Kingston, unpublished results) or ultrasound-assisted extraction [11] have been evaluated for different biological materials. Microwave and ultrasound extraction provide advantages over conventional extraction procedures in terms of time, extraction efficiency and solvent consumption by facilitating and accelerating the extraction process during the pretreatment of the biological samples.

The real efficiency of these methods is generally unknown because transformation of species is not assessed by standard extraction and sample preparation protocols. When combined with extraction recovery, both are masked in the combination. Using conventional analyses, critical comparison of these methods is difficult or impossible owing to the numerous sources of errors (not only extraction) that exist in mercury species determination. The comparison of extraction efficiencies is hindered by the differences in the other steps (derivatization, cleanup, etc.) and in the samples. The aim of this study was to evaluate these errors and to correct for them using newer analytical assessment techniques as described in the SIDMS protocol. Then, the second goal of this work was to apply the correction protocols for the comparison of the performance of various extraction procedures found in the literature to determine total mercury and mercury species using Tuna Fish Tissue Certified Reference Material (ERM-CE464) as a common sample. In the assessment, reagents and sample material were kept constant and analyses were carried out in the same clean laboratory and under the same conditions by a single analyst. With these conditions standardized, the performance of the overall methods could be reasonably attributed to variations in the efficiencies of the individual extraction methods [27]. EPA method 6800 (SIDMS) [17] was applied in this study as a definitive technique, as a correction tool for sample preparation and detection, and as an alternative overarching method for comparison of the speciation results.

Materials and methods

Instrumentation

An HP 4500 ICP-MS system (Agilent Technologies, Palo Alto, CA, USA, and Yokogawa Analytical System, Tokyo, Japan) was used in this work under the operational conditions summarized in Table 1. The sample delivery system consisted of a peristaltic pump and a quartz spray chamber with a concentric nebulizer and a quartz torch. The instrument, fitted with a nickel sampler and skimmer cones, was optimized daily using a 10 ppb tuning solution (Agilent Technologies, Palo Alto, CA, USA) containing Li, Y, Ce and Tl in 2% (v/v) HNO_3. The spectrum mode was engaged for direct analysis; the time resolved analysis mode was engaged for speciation analysis.

For HPLC separations, a polystyrene/divinylbenzene C18 reversed-phase column (150 mm×4.6 mm, 2 μm, Metrohm-Peak, Houston, TX, USA) was used. The HPLC column was equilibrated with at least 50 mL of mobile phase [50 mM pyridine, 0.5% (w/v) L-cysteine, 5% (v/v) methanol at pH 3] prior to the injection of mercury compounds. The chromatographic separation was carried out at room temperature with a flow rate of 1 mL/min.

Table 1 Inductively coupled plasma mass spectrometry (*ICP-MS*) and high-performance liquid chromatography (*HPLC*) operating conditions

Operational conditions of the HP-4500 ICP-MS system	
RF power	1,475 W
Plasma gas flow	Ar, 15 L/min
Auxiliary gas flow	Ar, 1 L/min
Nebulizer	Quartz, concentric
Spray chamber	Quartz
Sample and skimmer cones	Ni, 1.1 and 0.8 mm, respectively.
Measurement parameters of the HP-4500 ICP-MS system	
Monitoring isotopes	^{103}Rh[a], ^{199}Hg[a,b], ^{200}Hg[a,b], ^{201}Hg[a,b] and ^{202}Hg[a,b]
Acquisition mode	Spectrum[a] and time-resolved analysis[b]
Integration time per mass	0.25[a] and 0.20 s[b]
Replicates	5[a] and 1[b]
Total analysis time	24.5[a] and 300 s[b]
HPLC conditions	
Column	Polystyrene/divinylbenzene C18 reversed-phase column 150 mm×4.6 mm, 2 μm (Metrohm-Peak)
Injection volume	100 μL
Column temperature	Ambient
Mobile phase	50 mM pyridine, 0.5% (w/v) L-cysteine, 5% (v/v) MeOH, pH 3
Elution	Isocratic
Flow rate	1 mL/min

[a] For total mercury analysis
[b] For mercury speciation analysis

The HPLC unit consisted of an inert modular system equipped with a 709 IC pump, a 762 software interface and a 838 autosampler (Metrohm-Peak, Houston, TX, USA). The separation enclosure included a six-port injection valve fitted with a 100-μL sample loop. The solution eluted from the column was introduced to the ICP-MS system online by connecting short-length perfluoroalkoxy tubing to the nebulizer of the ICP-MS system.

An ETHOS 1 microwave laboratory system (Milestone, Sorisole, Italy) equipped with temperature and pressure feedback controls and magnetic stirring capability was used in this study for the digestion and extraction processes. This device is capable of sensing and controlling temperature to within ±1.0 °C of the set temperature and automatically adjusting the microwave field output power for temperature correction. This ETHOS 1 system can be used to extract/digest up to ten samples simultaneously. This particular system utilizes 100-mL TFM vessels.

A circulating water bath with a temperature range from 25 to 100 °C (model 260, Precision, Scientific Group, Chicago, IL, USA), an ultrasonic bath (Branson 2210, Danbury, CT, USA) and a hybridization oven with rotation (model 136500, Boekel Industries, Feasterville, PA, USA) were also used for the extraction processes. A FAM-40 vacuum unit (Milestone, Sorisole, Italy) was used to filter the digest and extracts. A centrifuge (model 225, Fisher Scientific, St. Louis, MO, USA) was employed for the sample preparation. A 0.01-mg Analytical Plus balance (Ohaus, Pine Brook, NJ, USA) was used in this work to weigh the samples and reference standards.

Reagents and materials

Analytical reagent grade HNO_3 and HCl (Fisher Scientific, Pittsburgh, PA, USA) were used. Double-deionized (DDI) water (18 MΩ cm) prepared from a Barnstead NANOpure water system (Dubuque, IA, USA) was used in the preparation of all solutions throughout this study. Reagent-grade TMAH, potassium hydroxide, optima-grade methanol and HPLC-grade glacial acetic acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). Reagent-grade L-cysteine, L-cysteine hydrochloride hydrate, ammonium phosphate monobasic, ammonium phosphate dibasic, pyridine and protease XIV (from *Streptomyces griseus*) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For the HPLC species separation, a mobile phase [50 mM pyridine, 0.5% (w/v) L-cysteine, 5% (v/v) methanol at pH 3], adapted from a published procedure [28], was prepared by diluting 4 mL pyridine, 5 g cysteine and 50 mL methanol in 950 mL DDI water. The pH of the solution was adjusted by adding concentrated HCl.

Samples and standards

Plastic and glass materials were cleaned by soaking them in 25% (v/v) HCl (24 h), then soaking them in 25% (v/v) HNO_3 (24 h) and finally rinsing them with NANOpure water. ERM-CE464 was supplied by the Institute for Reference Materials and Measurements (Geel, Belgium).

Natural abundance, enriched standard solutions and SIDMS software were provided as a commercially available mercury speciation kit by Applied Isotope Technologies (Sunnyvale, CA, USA). The concentrations of the natural abundance standards were 100 mg/L $HgCl_2$ in 1% (v/v) HNO_3 and 100 mg/L CH_3HgCl in water. Enriched standard solutions had nominal concentrations of 6.61±0.04 mg/L $^{199}HgCl_2$ (91.95% isotopic purity) in 1% (v/v) HNO_3 and 7.80±0.06 mg/L $CH_3{}^{200}HgCl$ (96.41% isotopic purity) in 1% $Na_2S_2O_3$, respectively. The internal standard rhodium was diluted from 10 mg/L in 2% HCl stock standard (High Purity Standards, Charleston, SC, USA).

All stock solutions of Hg^{2+} and CH_3Hg^+ were stored in precleaned amber glass vials in a cold room at 4 °C. Diluted working solutions were prepared by weight daily with proper dilution in 0.5% (v/v) HNO_3 (for total analysis) or in the mobile phase (for speciation analysis).

Procedures

Digestion procedure

For total mercury, ERM-CE464 was digested using EPA method 3052 [29]. Representative samples of approximately 0.2 g were weighed into microwave vessels, and 9 mL concentrated HNO_3 and 3 mL concentrated HCl were added to each vessel. The vessels were sealed and microwave-irradiated at 180±5 °C for 10 min. After digestion, the samples were filtered through a 0.22-μm Millipore glass fiber filter (Fisher Scientific, Pittsburgh, PA, USA). The samples were diluted with 0.5% (v/v) HNO_3 to a volume of 20 mL and stored in a cold room at 4 °C until analysis.

Extraction procedures

For mercury species, eight extraction procedures (herein denoted 1–8) were evaluated. Three subsamples of ERM-CE464 were prepared in each case. A procedural blank was prepared along with the samples for quality assurance purposes. After the extraction, the samples were stored in a cold room at 4 °C for less than 2 days before analysis.

Procedure 1 For alkaline extraction with 25% (w/v) KOH in methanol [11], a 300-mg portion of sample and 3 mL of 25% (w/v) TMAH in methanol were placed in a centrifuge tube. The mixture was heated in a 70 °C water bath for 30 min and sonicated for another 30 min. The procedure was repeated three times to properly digest the sample. After the extraction, the suspension was centrifuged at 3,500 rpm for 10 min. The digest was then transferred into a new centrifuge tube. Before the analysis, the pH of the digest was adjusted to 4 with 4 M acetic acid.

Procedure 2 For alkaline extraction with 25% (w/v) TMAH in methanol [11], the method was the same as method 1 with the exception that the methanol solution consisted of 25% (w/v) TMAH instead of 25% (w/v) KOH.

Procedure 3 For alkaline extraction with 5% (w/v) TMAH in methanol [12], a 200-mg portion of sample and 2 mL of 25% (w/v) TMAH in methanol were placed in a microwave vessel. The final volume was adjusted to 10 mL with ultrapure water for microwave requirements. The microwave program was as follows: step 1, room temperature to 180 °C, 10 min; step 2, 180 °C, 10 min. The vessels were then cooled to room temperature and the digest was filtered through a 0.22-μm glass fiber filter. Before the analysis, the pH of the digest was adjusted to 4 with 4 M acetic acid.

Procedure 4 For acid leaching with 5 M HCl [11], a 300-mg portion of sample and 5 mL of 5 M HCl were placed in a centrifuge tube and sonicated for 5 min in an ultrasonic bath. After the extraction, the suspension was centrifuged at 3,500 rpm for 10 min. The digest was then transferred to a new centrifuge tube. Before the analysis, the pH of the digest was adjusted to 4 with 4 M acetic acid.

Procedure 5 For microwave-assisted extraction, method 3200 was used [30] (G.M.M. Rahman, T. Fahrenholz and H.M. Kingston, unpublished results). A 500-mg portion of sample was weighed into individual precleaned Teflon digestion vessels. Then, 10 mL of 4 M HNO_3 was placed in each of the microwave extraction vessels. A magnetic stir bar was added to each vessel for thorough mixing of solvent with the sample. The microwave vessels were sealed and irradiated at 100 °C for 10 min with magnetic stirring. A 2-min ramping time was used to reach the desired temperature of 100 °C. After microwave irradiation, the vessels were cooled to room temperature and the extract was filtered through a 0.22-μm glass fiber filter. Before the analysis, the pH of the extract was adjusted to 4 with 4 M acetic acid.

Procedure 6 For acid leaching with glacial acetic acid [13], a 300-mg portion of sample and 9 mL of glacial acetic acid were placed in a vessel container. The microwave program was as follows: step 1, room temperature to 165 °C, 2 min; step 2, 165 °C, 8 min. The vessels were then cooled to room temperature and the digest was filtered through a 0.22-μm glass fiber filter. The digest was then transferred to a new

centrifuge tube. Before the analysis, the pH of the digest was adjusted to 4 with 4 M acetic acid.

Procedure 7 For 1% (w/v) L-cysteine hydrochloride extraction [14], a 200-mg portion of sample and 20 mL of 1% (w/v) L-cysteine hydrochloride hydrate were added to the extraction vial. The vials were capped tightly, shaken vigorously by hand for 15–20 s and then placed for 2 h in a water bath maintained at 60 °C. The vials were shaken vigorously by hand for 15–20 s two more times, after 1 and 2 h of heating. The vials were cooled to room temperature by placing them in water at 22 °C for 10–15 min. The extracts were filtered through a 0.22-μm glass fiber filter. Before the analysis, the pH of the extract was adjusted to 4 with 4 M acetic acid.

Procedure 8 For enzymatic digestion with protease XIV [15], A 200-mg portion of sample was weighed in a 10-mL glass culture tube with 20 mg of protease type XIV and 8 mL of 0.1 M phosphate buffer (pH 7.5) containing 0.05% (w/v) cysteine. The tubes were incubated for 2 h in a hybridization oven at 37 °C while being spun at 20 rpm. The extracts were transferred to acid-washed polypropylene centrifuge tubes, the final volume was adjusted to 10 mL with buffer and the extracts were centrifuged for 20 min at 3,000 rpm. Supernatants were filtered through 0.22-μm glass fiber filters. Before the analysis, the pH of the extract was adjusted to 4 with 4 M acetic acid.

Analysis of the extracts by ICP-MS and HPLC-ICP-MS

The prepared solutions were further diluted and analyzed by ICP-MS and HPLC-ICP-MS.

Total mercury determination by ICP-MS For measurement of total mercury concentrations, the digested (EPA method 3052) and extracted solutions were analyzed by ICP-MS in spectrum mode. The plasma parameters used for the analysis are summarized in Table 1. Total mercury concentrations were determined by external calibration. Calibration was performed by using 1–20 μg/L Hg^{2+} standards in 0.5% (v/v) HNO_3. All standards and samples were spiked with 5 μg/L rhodium as an internal standard. The most abundant mercury isotope, *m*/*z* 202, was used for data evaluation; similar results were obtained using the mercury isotopes *m*/*z* 199, 200 and 201.

Mercury species determination by HPLC-ICP-MS Analyses were performed in time resolved analysis mode. The experimental conditions are given in Table 1. Each sample was analyzed four times to enable statistical evaluation of the samples ($n=3\times4$). Data evaluation was performed using the ChemStation software supplied with the instrument, and quantification was based on peak areas by external calibration. The most abundant mercury isotope, *m*/*z* 202, was used for data evaluation; similar results were obtained using the mercury isotopes *m*/*z* 199, 200 and 201.

SIDMS extraction procedure

In order to perform SIDMS analysis of each of the selected mercury extraction methods, ERM-CE464 was weighed into either centrifuge tubes or microwave vessels (based on the corresponding method requirement), and suitable amounts of $^{199}Hg^{2+}$ and $CH_3{}^{200}Hg^{+}$ were added to each tube/vessel so as to provide a ratio of measured isotopes close to unity and minimize random error propagation. The samples were allowed to equilibrate for 1 h and were then extracted according to the procedure discussed for each selected method. Extracts were analyzed using HPLC-ICP-MS. The experimental conditions are given in Table 1.

Peak areas were used to calculate isotopes ratios $^{199}Hg/^{202}Hg$ and $^{200}Hg/^{202}Hg$, from which the Hg^{2+} and CH_3Hg^{+} concentrations in ERM-CE464 were calculated. All isotope ratios were corrected for the detector dead time, and a natural abundance standard solution of Hg^{2+} and CH_3Hg^{+} was measured periodically between samples to calculate the mass bias correction factor. Further, this double-spike approach allowed tracking of any artifact methylation/demethylation reactions that occurred during the sample preparation and/or analysis process. The calculations were carried out using the software provided by Applied Isotope Technologies. The method 6800 SIDMS protocol uses direct mathematical algorithmic solutions for isotope ratio calculations and quantitative determinations instead of conventional calibration curves. These direct solutions were used as specified in EPA method 6800 for all species calculations. All statistical calculations, including one-way analysis of variance, were performed using Microsoft Excel 2003 software. A significance level of $P < 0.05$ was adopted for all comparisons.

Results and discussion

Total mercury content in ERM-CE464 was determined using EPA method 3052 [29] to evaluate the levels of error for different analytical procedures used for species extraction. A total mercury concentration of 5.28±0.36 mg/kg for $n=3$ at the 95% confidence level was obtained. The recovery of mercury was satisfactory in the range from 93 and 109% of the certified value (5.24±0.10 mg/kg).

Evaluation of the total mercury extraction efficiency

The aim of this study was to evaluate the extraction efficiency of eight selected methods used for total mercury

and mercury speciation analyses in biological matrices. The extraction efficiencies in the methods tested are generally unknown because transformation of species is not assessed by standard extraction, sample preparation protocols and analyses. The performance of these extraction methods was evaluated by analysis of ERM-CE464 with certified values for total mercury and CH_3Hg^+, respectively. Fish and other seafood products are a main source of CH_3Hg^+ in the diet [2, 3]. The primary chemical form of CH_3Hg^+ in fish muscle has been identified as a CH_3Hg–cysteine compound, which is likely to be incorporated in larger proteins [31].

These eight extraction procedures were somewhat independent and unique in their approaches to sample extraction of the mercury species (Table 2). Procedures 1–3 are based on the traditional alkaline extraction, which is better described as saponification (breakdown of fats into their corresponding fatty acids) [19, 25]. Procedures 1 and 2 used ultrasound-assisted extraction with 25% (w/v) KOH in methanol or 25% (w/v) TMAH in methanol, respectively. In procedure 3, microwave-assisted extraction using 5% (w/v) TMAH in methanol was evaluated. Furthermore, acid leaching using ultrasound-assisted extraction with 5 M HCl (procedure 4) and a microwave-assisted extraction technique with either 4 M HNO_3 (procedure 5) or glacial acetic acid (procedure 6) were tested. Conversely, procedure 7 is a conventional heating method using 1% (w/v) L-cysteine hydrochloride. Since mercury has high affinity for the sulfydryl group, one kind of sulfydryl reagent is used in many studies as a component of the extraction system [14, 23, 24]. Finally, procedure 8 is based on enzymatic digestion with protease XIV. The enzymatic digestion has the advantage that the enzymes act only on specific chemical bonds and are not likely to alter the chemical form of the mercury species [15]. Among the procedures, the use of 5 M HCl (procedure 4) resulted in the fastest method, compared with procedures 1 and 2 [alkaline leaching with KOH 25% (w/v) in methanol and TMAH 25% (w/v) in methanol, respectively], which both require 3 h.

The samples were quantified using ICP-MS for total mercury determination and HPLC-ICP-MS for mercury speciation analysis. The results for total mercury and mercury speciation analyses in ERM-CE464 are summarized in Table 3. The results are provided as averages of three different replicates. To assess total mercury and CH_3Hg^+ recovery, the concentrations of mercury by ICP-MS or CH_3Hg^+ by HPLC-ICP-MS were compared with those corresponding to the certified value. These values are given in parentheses.

It can be seen from Table 3 that the results for total mercury concentrations between ICP-MS measurements and the sum of the species obtained by HPLC-ICP-MS were statically indistinguishable by the ANOVA one-way test ($P>0.05$), except for procedure 7. In procedure 7, the sum of the species by HPLC-ICP-MS resulted in higher values than the total mercury results by ICP-MS owing to trace mercury contamination in the cysteine hydrochloride reagent used. As reported in the literature [14], trace Hg^{2+} contamination of the reagents significantly affects the background and noise of the HPLC chromatograms. The Hg^{2+} concentration in 1% (w/v) L-cysteine hydrochloride calculated by external calibration was approximately 0.024 μg/L.

For total mercury, no significant difference (t test, $P < 0.05$) was found between the certified value and the ones provided by procedures 1 and 2 [alkaline extraction using 25% (w/v) KOH in methanol or 25% (w/v) TMAH in

Table 2 The extraction procedures evaluated

Procedure	Extraction reagent	Extraction technique/extraction temperature	Total extraction time (min)	References
1	25% (w/v) KOH in MeOH	Sonication bath/water bath/70 °C	180	[11]
2	25% (w/v) TMAH in MeOH	Sonication bath/water bath/70 °C	180	[11]
3	5% (w/v) TMAH in MeOH	Microwave /180 °C	20	[12]
4	5 M HCl	Sonication bath/room temperature	5	[11]
5	4 M HNO_3 (EPA method 3200)	Microwave/180 °C	20	[30] and G.M.M. Rahman, T. Fahrenholz and H.M. Kingston (unpublished results)
6	Glacial CH_3COOH	Microwave/165 °C	10	[13]
7	1% (w/v) L-cysteine hydrochloride	Water bath/60 °C	120	[14]
8	Enzymatic digestion with protease XIV	Hybridization oven/37 °C	120	[15]

TMAH tetramethylammonium hydroxide

Table 3 Total mercury concentrations and mercury speciation analysis in the extracts of Tuna Fish Tissue Certified Reference Material (ERM-CE464) measured by ICP-MS and HPLC-ICP-MS, respectively

Procedure	ICP-MS		HPLC-ICP-MS	
	Total Hg, (mg/kg)$^{-}$	Hg^{2+} (as Hg), (mg/kg)	CH_3Hg^+ (as Hg) (mg/kg)	Sum of species (mg/kg)
1	5.24±0.34 (100±6)	0.06±0.02	5.05±0.13 (99±3)	5.11±0.13 (98±3)
2	5.19±0.59 (99±6)	0.12±0.03	5.05±0.18 (99±4)	5.17±0.18 (99±3)
3	4.82±0.20 (92±4)	0.18±0.05	4.88±0.17 (95±3)	5.06±0.18 (97±3)
4	4.25±0.49 (81±9)	0.07±0.02	4.29±0.39 (84±8)	4.36±0.39 (83±7)
5	4.00±0.13 (76±2)	0.06±0.04	3.94±0.12 (77±2)	4.00±0.13 (76±2)
6	3.62±0.47 (69±9)	0.35±0.08	3.29±0.14 (64±3)	3.64±0.16 (69±3)
7	4.58±0.43 (87±8)	0.45±0.10	4.87±0.20 (95±4)	5.32±0.22 (102±4)
8	4.60±0.55 (88±10)	0.16±0.07	4.42±0.14 (86±3)	4.58±0.16 (87±3)

The results were obtained by external calibration. The values are means±95% confidence level (n=3)
Certified total Hg is 5.24±0.10 mg/kg, certified CH_3Hg^+ is 5.12±0.16 mg/kg and estimated Hg^{2+} is 0.12 mg/kg. The percentage recoveries of total Hg and CH_3Hg^+ are indicated in *parentheses*.

methanol]; the mercury extraction recoveries were 100±6 and 99±6%, respectively. These values were higher than those observed by Cabañero-Ortiz et al. [11] when comparing these two extraction procedures. Application of the method of Cabañero-Ortiz et al. resulted in the extraction of approximately 64% and approximately 67% of total mercury using KOH/methanol and TMAH/methanol, respectively, from an oven-dried tuna fish sample.

Mercury extraction recoveries were lower for procedures 3, 4, 7 and 8. The total mercury recovery ranged from 81% in procedure 4 to 92% in procedure 3. For procedures 3, 4, 7 and 8, there was not a significant distinguishable difference between these lower recoveries as demonstrated by the use of the one-way ANOVA test (P>0.05).

The lowest extraction recoveries of total mercury were observed using procedures 5 and 6 (microwave extraction using 4 M HNO_3 and acetic acid, respectively). The total mercury recoveries were 76±2 and 69±9%, respectively.

While alkaline and acid leaching are currently two of the most widely used procedures for the extraction of mercury species from biological samples, lower extraction efficiencies of total mercury were obtained, in this work, with the procedures based on acid leaching extraction (procedures 4–6). These procedures are based on the use of 5 M HCl (procedure 4), 4 M HNO_3 (procedure 5) or acetic acid (procedure 6). With respect to the acid extraction with 5 M HCl, an extraction recovery of 80±9% was obtained. The results for procedure 4 were less than the reported value by the original method [11]. The extraction efficiency of mercury reported by Cabañero-Ortiz et al. was 97±5%. However, HCl was more efficient in the liberation of protein-bound mercury species than 4 M HNO_3 or acetic acid extraction reagents.

Evaluation of the extraction efficiency of mercury species

The chromatographic method used for this analysis is based on the separation of Hg^{2+} and CH_3Hg^+ as cysteine–mercury complexes on a polymeric-based C18 reversed-phase column [28]. The chromatographic conditions are reported in Table 1. The chromatogram of Hg^{2+} and CH_3Hg^+ in aqueous standard solution (10 μg/L as Hg) using the optimal operating conditions is shown in Fig. 1. The separation was achieved in less than 5 min and the retention times were 1.87±0.02 and 2.98±0.03 min, respectively. The calibration curves based on peak area were linear for both species in the range from 1 to 20 μg/L. The observed detection limits of ^{202}Hg (calculated as 3σ of the baseline noise, based on peak height) were 0.46±0.02, and 0.78±0.08 μg/L for Hg^{2+} and CH_3Hg^+, respectively.

The results of mercury speciation analysis of ERM-CE464 by HPLC-ICP-MS are given in Table 3. Only procedures 1 and 2, based on the use of alkaline leaching, provided results statistically similar to the certified value (t test, P>0.05). The CH_3Hg^+ recoveries were 99±3 and 99±4%, respectively. The results in Table 3 show quantitative CH_3Hg^+ recoveries for procedures 3 [alkaline extraction with 5% (w/v) TMAH using microwave extraction) and 7 (extraction using 1% (w/v) L-

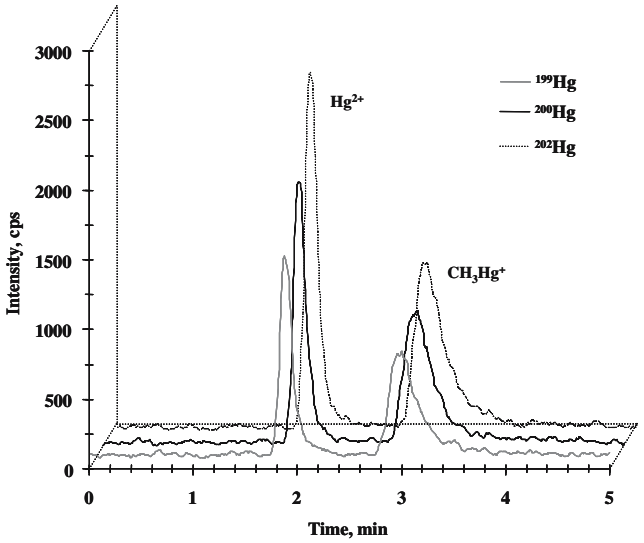

Fig. 1 High-performance liquid chromatography–inductively coupled plasma mass spectrometry chromatogram for 10 μg/L Hg^{2+} and CH_3Hg^+. Chromatograms obtained at different masses were shifted for clarity. The chromatographic conditions are given in Table 1

cysteine hydrochloride]. The CH_3Hg^+ recoveries were 95±3 and 95±4%, respectively. Although these recoveries were only slightly lower than those provided by procedures 1 and 2, they were statically different from the certified value (*t* test, $P < 0.05$). While extraction procedure 7 was suitable for CH_3Hg^+ determination in ERM-CE464, trace Hg^{2+} contamination was found in the L-cysteine hydrochloride reagent and usage of a large amount of reagent during the pretreatment of the sample resulted in increased blank values and higher detection limits.

Lower CH_3Hg^+ recoveries were observed when 5 M HCl (procedure 4) and protease XIV (procedure 8) were used. CH_3Hg^+ recoveries were 84% for procedure 4 and 86% for procedure 8. Cabañero-Ortiz et al. [11] reported 98±7% CH_3Hg^+ recovery in tuna fish certified material from the Institute for Reference Materials and Measurements (CRM-433) during the development of procedure 4. No Hg^{2+} data were reported in that work. For procedure 8, similar results (90±2%) were reported for dogfish muscle (DORM-2) from the National Research Council of Canada when the extraction procedure was developed [15]. For acid leaching methods, only 77±2 and 64±3% of CH_3Hg^+ were recovered using microwave-assisted extraction techniques with 4 M HNO_3 (procedure 5) and acetic acid (procedure 6), respectively. Although the use of 4 M HNO_3 (EPA method 3200) has produced satisfactory results for the extraction of mercury species in samples such as sediments [30] or human hair (G.M.M. Rahman, T. Fahrenholz and H.M. Kingston, unpublished results), the CH_3Hg^+ recovery obtained for tuna fish was unsatisfactory. With respect to the acetic acid leaching protocol [13], CH_3Hg^+ in a variety of biological reference materials was evaluated, including Mussel Tissue (SRM 2977), Oyster Tissue (SRM 1566b) and Lake Superior Fish Tissue (SRM 1946) from the National Institute of Standards and Technology. However, in different applications of this protocol by researchers such as Tseng et al. [32], the results were more consistent with the lower recoveries found in this study. These findings of these authors and our findings suggest that lower recoveries may be due to insufficient dissolution of difficult biological tissues by acetic acid.

During the evaluation of Hg^{2+} concentration for the extraction methods, lower precision in these results was observed. The certified reference material (ERM-CE464) is not certified for Hg^{2+}, but is only certified for CH_3Hg^+ and total mercury; consequently, the concentration of Hg^{2+} was calculated from the difference in these two values. This fish tissue material has low concentrations of Hg^{2+}, and these were determined by difference to be approximately 0.12 μg/g in the solid certified reference material and represents only 2.3% of the total mercury concentration in the solid sample. Low concentration, noncertified values and high uncertainties in the values for Hg^{2+} did not yield statistically meaningful comparisons between extraction methods.

Evaluation of selected mercury speciation methods using SIDMS

The application of SIDMS analysis (EPA method 6800) depends on method-specific fundamental operations: isotopic spike preparation and calibration or purchase of an isotopic spike analogue; sample collection and sample spiking; sample species and spike species equilibration; sample extraction; species separation; isotope ratio measurements of each speciated component; determination of species concentrations, mathematical deconvolution of species transformations and application of these corrections.

In order to perform the SIDMS analysis, a known amount of ERM-CE464 was double-spiked with known amounts of isotopically enriched Hg^{2+} ($^{199}Hg^{2+}$) and CH_3Hg^+ ($CH_3{}^{200}Hg^+$) in such a way that the desired isotope ratios $^{199}Hg/^{202}Hg$ and $^{200}Hg/^{202}Hg$ became close to unity at complete equilibration. After equilibration with the sample species, the samples were extracted and analyzed by HPLC-ICP-MS. The dead time and mass bias corrected isotope ratios for $^{199}Hg/^{202}Hg$ and $^{200}Hg/^{202}Hg$ were calculated for both Hg^{2+} and CH_3Hg^+ in each of the sample replicates. SIDMS calculations were performed to determine the concentration of Hg^{2+} and CH_3Hg^+, as well as to deconvolute the interspecies transformations using the SIDMS software provided by Applied Isotope Technologies. The detailed description of data processing and application of the SIDMS software algorithms were published previously [33, 34].

The final concentrations of Hg^{2+} and CH_3Hg^+ in ERM-CE464 and the percentage mercury species transformation

during the extraction procedures are summarized in Table 4. The sum of the mercury species was calculated and the percentage of recovery with respect to the certified values can also be evaluated.

It can be seen in Table 4 that the CH_3Hg^+ values found by using SIDMS (EPA method 6800) for seven of the eight extraction procedures evaluated (with the exception of procedure 5) were in good agreement with the certified reference value (*t* test, $P>0.05$). CH_3Hg^+ recoveries were corrected to within a range of 99–102%. For extraction procedure 5, the CH_3Hg^+ recovery was 109±6%. As expected, the SIDMS protocol was able to overcome nonquantitative recoveries and species transformations observed during the evaluation of the extraction procedures.

The percentages of methylation of $^{199}Hg^{2+}$ and demethylation of $CH_3{}^{200}Hg^+$ obtained using the extraction methods studied are shown in Table 4. When HPLC separation is used, the transformation and losses of Hg^{2+} and CH_3Hg^+ might be directly linked to pretreatment steps.

For methylation, the results for most of the procedures were not significantly different (one-way ANOVA test, $P>0.05$). Methylation factors were within the range 3– 6%. The largest amount of methylation was observed for procedure 5 (microwave extraction using 4 M HNO_3), in which the percentage of methylation was as high as 18%. Owing to the relatively low ratio of Hg^{2+} to CH_3Hg^+, high methylation did not cause a significant error when external calibration was used. However, caution should be exercised with biological samples in which the ratio of CH_3Hg^+ to Hg^{2+} is low. It can be observed from Table 4 that demethylation occurred in all the extraction procedures evaluated. As a consequence, for determination of CH_3Hg^+ and Hg^{2+} concentrations, both reactions should be considered, particularly if both species are present in a similar concentration range. Conversion of CH_3Hg^+ to Hg^{2+} resulting from the application of procedure 5 was negligible. Demethylation factors for procedures 4 and 8 were statistically indistinguishable by the ANOVA one-way test ($P>0.05$): 3% for HCl extraction and 1.5% for enzymatic digest with protease, respectively. However, for procedures 1–3 and 7, degradation factors of up to 6% were observed. They were statically indistinguishable (one-way ANOVA test, $P>0.05$). Acid leaching using glacial acetic acid (procedure 6) caused the highest degree of conversion from CH_3Hg^+ to Hg^{2+}, where the degradation factor increased to 27%. The higher measured value of Hg^{2+} observed with extraction procedure 6 (microwave digestion using acetic acid) might reflect the influence of a potential demethylation transformation reaction occurring in this procedure.

On the basis of experimental design and the data shown in Tables 3 and 4, it was confirmed that alkaline digestion methods are suitable for quantitative recovery of CH_3Hg^+ from biological samples, including foods and tissues, using either a microwave device (procedure 3) or an ultrasonic extraction system (procedures 1 and 2) with equivalent conversions between mercury species compared with other extraction methods; however, the required extraction time was significantly reduced using the microwave extraction procedure (procedure 3). Although similar results were obtained with procedure 7, Hg^{2+} contamination in the extraction reagent was observed. Procedures 4 and 8 produced less transformation of CH_3Hg^+ to Hg^{2+}, but the extraction recovery of these two methods was lower than the recoveries from procedures 1–3 and 7. Owing to the highest species transformations found in procedures 5 and 6, the application of these two extraction procedures for mercury

Table 4 Deconvoluted concentration and estimated degree of mercury species transformation in ERM-CE464 during the extraction procedures evaluated (values are means±95% confidence level) by speciated isotope dilution mass spectrometry HPLC-ICP-MS

Extraction procedure	EPA method 6800 (HPLC-ICP-MS)				
	Hg^{2+} (mg/kg)	CH_3Hg^+ (mg/kg)	Sum of species (mg/kg)	Mean degree of transformation±95% confidence level (%)	
				Hg^{2+} to CH_3Hg^+	CH_3Hg^+ to Hg^{2+}
1	0.07±0.02	5.22±0.31 (102±6)	5.29±0.31 (101±6)	5±3	6±1
2	0.07±0.03	5.20±0.18 (102±4)	5.27±0.18 (101±6)	6±2	4±1
3	0.30±0.07	5.18±0.13 (101±3)	5.48±0.15 (105±3)	3±2	6±2
4	0.13±0.05	5.11±0.38 (100±7)	5.24±0.38 (100±7)	5±3	3±1
5	0.11±0.07	5.60±0.33 (109±6)	5.71±0.34 (109±6)	18±4	0.8±0.6
6	0.27±0.12	5.12±0.19 (100±4)	5.39±0.22 (103±4)	4±2	27±5
7	1.05±0.14	5.08±0.25 (99±5)	6.13±0.29 (117±5)	4±3	4±1
8	0.15±0.05	5.09±0.24 (99±5)	5.24±0.25 (100±5)	4±2	1.4±0.5

Certified total Hg is 5.24±0.10 mg/kg, certified CH_3Hg^+ is 5.12±0.16 mg/kg and estimated Hg^{2+} is 0.12 mg/kg. The percentage recoveries of total Hg and CH_3Hg^+ are indicated in *parentheses*.

speciation analysis in biological tissues will cause overestimation/underestimation of the concentration of mercury species without being paired with EPA method 6800.

For these extraction protocols, by using the isotope dilution technique, the amount of mercury species conversion which took place during extraction was accounted for, so an accurate determination of CH_3Hg^+ in biological samples could be made.

Conclusions

Different extraction methods for mercury speciation in food and biological samples such as ERM-CE464 were evaluated using both conventional and SIDMS techniques. On the basis of the results of this study, the methods based on the use of alkaline reagent (procedures 1–3) provide the highest extraction recoveries for total mercury and CH_3Hg^+ and are more suitable for mercury speciation using conventional techniques. However, both Hg^{2+} methylation and CH_3Hg^+ demethylation were observed and should be considered to be usually present, particularly if both species are present in a similar concentration range. The average relative errors of the CH_3Hg^+ determination by conventional methods were found to be approximately 13%, with a maximum bias of 36%; these errors were corrected to an average of less than 2% of the certified value by applying the EPA method 6800 protocol. The dynamic capabilities of SIDMS were demonstrated for correction of extraction errors and for minimization of errors in species quantitation. Direct mathematical protocols instead of conventional calibration clearly demonstrate that accuracy can be improved by using enriched isotope species labels to correct for these species transformations at the sample preparation and quantitative stages of analyses.

Acknowledgements We gratefully acknowledge Milestone Inc. for financial support to L.H.R. and for the provision of the microwave unit employed in this work. We also thank Agilent Technologies, Applied Isotope Technologies, Inc., Duquesne University and Metrohm-Peak, LLC for instrumental and material support. Portions of this method are patented or patent pending.

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